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(71) Applicant: GEORGETOWN UNIVERSITY [US/US]; Ryan Administration Building, 37th & O Streets, N.W., Washington, DC 20057 (US).

(72) Inventors: RAHMAN, Aquilur ; 13504 Bonniedale Drive, Gaithersburg, MD 20878 (US). THIERRY, Alain ; 2611 Dumbarton Street, N.W., Washington, DC 20007 (US). DRITSCHILO, Anatoly ; 8107 Fenway Road, Bethesda, MD 20817 (US).

(74) Agents: GNUSE, Robert, F. et al.; Oblon, Spivak, McClelland, Maier & Neustadt, Crystal Square Five - Fourth Floor, 1755 South Jefferson Davis Highway, Arlington, VA 22202 (US).

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(54) Title: METHOD OF ENCAPSULATING ANTHRACYCLINE GLYCOSIDES IN LIPOSOMES

(57) Abstract

A method of preparing a liposomal anthracycline glycoside composition, which entails forming cardiolipin-containing liposomes by drying a lipid mixture containing cardiolipin, and then introducing an aqueous solution, and mixing the cardiolipin-containing liposomes with a solution of anthracycline glycoside.

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Description

METHOD OF ENCAPSULATING ANTHRACYCLINE GLYCOSIDES IN LIPOSOMES

Technical Field

The present invention relates to a method of encapsulating anthracycline glycosides in liposomes and liposomal compositions obtainable thereby.

Background Art

Anthracycline glycosides are compounds which are known to exhibit both antibiotic and anticancer activity. Generally, anthracycline glycosides contain an amino-sugar linked to a tetrahydronaphthacene chromophore by a glycoside bond. Among this group of compounds are doxorubicin (adriamycin, a product of U.S. Patent 3,590,028) and daunorubicin (U.S. Patent 4,012,284) as well as analogs described in U.S. Patent 3,686,136 and Yamamoto, K. et al., J. Med. Chem. 15, 872 (1973); German patent nos. 2,327,211; 2,557,537; and 1,920,198; Bachman, E. et al., Agents and Actions, 5/4, 383, 1975); Chandra, P., Cancer Chemotherapy, Rep. 6,115 (1975); Arcamone, F. et al., id. at 123; and Zbinden, G. et al., Cancer Chemotherapy, Rep. 4, 707 (1975).

Doxorubicin is one of the most commonly used antineoplastic agents and has demonstrated activity for a wide range of human cancers such as leukemia, lymphoma and solid tumors. The mechanisms of action of anthracycline glycosides are considered to involve the blocking of the functions of deoxyribonucleic acid (DNA) by intercalation in the DNA structure. Unfortunately, clinical use of these compounds is severely hampered by dose-limiting cardiotoxic effects. The cardiotoxicity of both doxorubicin and daunorubicin has been well documented. Further, the cardiotoxic effect has been shown to be cumulative and may lead to congestive heart failure. This cardiotoxicity is quite specific and pharmacologic distribution studies have shown a selective tropism to the heart muscle.

Attempts to prevent anthracycline glycoside cardiotoxicity have included reliance on combination chemotherapy to produce additive or synergistic effects so that the cumulative dosage of the anthracycline glycosides can be reduced, and concomitant administration of antioxidants to protect against cardiotoxic effects.

More recently, liposome delivery systems have been used to reduce anthracycline uptake in cardiac tissue, while preserving drug activity. See for example, U.S. Patent 4,419,348. In using this methodology, it is found that doxorubicin in cardiolipin containing liposome exerts antitumor activity at doses that cause fewer myocardial alterations than the same dose of free doxorubicin. For example, prevention of doxorubicin cardiotoxicity in beagles by liposomal encapsulation has also been shown. Generally, the preparation of this form of doxorubicin involves a two--step preparation process, which entails complexing of the drug with cardiolipin and subsequently encapsulating the complex in liposomes. However, this preparation is very difficult to manage at an industrial level, particularly in conserving good encapsulation efficiency and preserving anthracycline glycoside stability.

Hence, a need exists for a method of preparing liposomal doxorubicin compositions which conserves good encapsulation efficiency while preserving anthracycline glycoside stability.

Disclosure of the Invention

Accordingly, it is an object of the present invention to provide a method for producing a anthracycline glycoside composition which affords good encapsulation efficiency.

It is also an object of the present invention to provide a method for producing a anthracycline glycoside composition which preserves anthracycline glycoside stability.

It is also an object of the present invention to provide a method for preparing a liposomal anthracycline glycoside composition, which is, at once, simple and straightforward.

Accordingly, the above objects and others which will become apparent in view of the following disclosure are provided by a method of preparing a liposomal anthracycline glycoside composition, which entails forming cardiolipin-containing liposomes by drying a lipid mixture containing cardiolipiry, and then introducing an aqueous solution, and b) mixing the cardiolipin-containing liposomes with a solution of anthracycline glycoside.

Brief Description of the Drawings

Figure 1 illustrates evaluation of liposome-doxorubicin complex relative to the time of incubation at 25°C following the mixing of both components.

Figure 2 illustrates a comparison of cytotoxicity of free doxorubicin, liposome-encapsulated doxorubicin and cardiolipin-liposome-complex doxorubicin using LZ cells.

Figure 3 illustrates survival curves of MCF-7/ADR cells treated with free doxorubicin, empty liposomes, cardiolipin-liposomes and doxorubicin in simultaneous combinations with empty cardiolipin-liposomes.

Figure 4 illustrates the reversal capacity of multidrug resistance by various treatments in LZ cells with liposome-encapsulated doxorubicin in LZ cells.

Best Mode for Carrying Out the Invention

In accordance with the present invention, it has been discovered that anthracycline glycosides may be encapsulated within liposomes in a surprisingly effective manner. The

encapsulated anthracycline glycosides may then be injected into a mammalian host to effectively kill cancer cells with a reduced toxicity to the host body. In accordance with the present invention, any one or more anthracycline glycosides may be encapsulated. For example, actinomycin as described in U.S. Patent 3,993,754, cis-platen analog as described by Perez-Solar R. Khokar Ar, Lopez-Berestein G. Treatment in Prophylaxis of Experimental Liver Metastases of M5076 Reticulosarcoma With Cis-Bisneodecanaoto-trans-R-1,2-diaminocyclohexane platinum (II) Encapsulated in Multi-lamellar Vesicles, Cancer Research 1987, 47:64-62-6 and doxorubicin as described in U.S. Patent 4,419,398 may be so encapsulated to exhibit reduced inherent toxicities while preserving antitumor activity.

In accordance with the present invention, by the term "liposomes" is meant a closed structure composed of lipid bi-layers surrounding an internal aqueous space. It appears that the decreased cardiotoxicity when using liposomalencapsulated doxorubicin, for example, is attributable to the fact that the liposomes do not accumulate in the heart in as great of a concentration as the free drug is believed to accumulate.

The present invention is, in part, directed to a liposomal anthracycline glycoside preparation which is based on a composition of one or more anthracycline glycosides and cardiolipin-containing liposomes. The present invention also pertains to a method of preparing such a composition.

Generally, the one or more anthracycline glycosides of the present invention may be any which exhibits antibiotic and/or oncolytic activity and which is characterized by cardiac tissue uptake in a mammalian host and by attendant cardiotoxicity and/or cardiomyopathy. Additionally, non-toxic and pharmaceutically acceptable analogs, derivatives or salts thereof may also be used. Further, as it is well-documented

that doxorubicin hydrochloride (adriamycin) and daunorubicin are such anthracycline glycosides, they may also be used in the present invention. Thus, the present invention is explicitly limited to any specific embodiment of anthracycline glycoside.

Further, it is specifically within the ambit of the present invention to use analogs of anthracycline glycosides. Analogs of anthracycline glycosides which are used as anticancer agents are well known to those skilled in the art. In accordance with the present invention, any analog of an anthracycline glycoside may be used, which is presently used as an anticancer agent. For example, deoxy analogs, such as 7-deoxy-doxorubicin may be used. Epirubicin may also be used.

In accordance with the present invention, the cardiolipin-containing liposomes may be formed by drying a lipid mixture containing cardiolipin and then introducing an aqueous solution. Dispersion is completed by strongly homogenizing the mixture using a vortex, magnetic stirrer and/or sonication. The liposomes are negative liposomes considering the inherent negative charges of cardiolipin and that the lipid added to cardiolipin must be neutral or negative such as phosphatidyl choline, cholesterol, phosphatidyl serine or phosphatidyl glycerol. For better stability, however, the liposome preparation may be lyophilized.

In accordance with the present invention, the cardiolipin used may be obtained from either a natural or synthetic source as is known to those skilled in the art.

The present invention is, in part, predicated upon the discovery that anthracycline glycosides may be complexed to the cardiolipin-containing liposomes prior to clinical administration. Surprisingly, complexation is achieved by simply mixing cardiolipin-containing liposomes concentrate

with a solution of anthracycline glycoside or by rehydration. For example, doxorubicin may be diluted in a 0.90% NaCl solution with 0.0017 M phosphate buffer at a ph.of 7.4.

The anthracycline composition may be prepared also by two additional methods: 1) by rehydrating lyophilized cardiolipin containing liposomes with a solution of anthracycline glycoside, or 2) by rehydrating with the buffer the mixture of lyophilized liposomes and crystalline doxorubicin.

The two additional methods described above may be practiced as follows. First, lyophilized cardiolipin-containing liposomes may be rehydrated with a solution of anthracycline glycoside. The cardiolipin-containing liposomes may be lyophilized using conventional methodologies. The rehydrating solution of anthracycline glycoside will have a concentration of at least about 15 μg/ml. Of course, all solutions described in the present specification are in pharmaceutically-acceptable solvents, such as saline solution of physiologically-acceptable concentration and pH and dextrose 5% saline.

Second, the present compositions may be prepared by rehydrating with buffer a mixture of lyophilized liposomes and crystalline doxorubicin. The buffer used may be those as generally described in the present specification.

Complexation of the anthracycline glycoside drug to cardiolipin-containing liposomes is due to the high binding affinity of such drugs, such as doxorubicin, to cardiolipin. From a pharmacological standpoint, the molar ratio of anthracycline glycoside to lipids may be adapted according to the type of tumor cells to be treated or the therapeutic end-point required. However, in order to obtain a good complexing efficiency, the cardiolipin content in liposomes should be at least half that of anthracycline glycoside added in terms of molar ratio. The complexed anthracycline glycosidecardiolipin

is strongly stabilized by an electrostatic interaction between two molecules of the glycoside and one molecule of cardiolipin and a stoichiometric interaction leading to a card pack dimer formation.

This strong binding allows for the insertion and the combination of the drug in the membrane lipids bilayer. Hence, the anthracycline glycoside is not situated at the surface of the liposome after complex formation but, rather, is firmly integrated in the liposome, which may then provide for a drug delivery that is as efficient as liposome-encapsulated anthracycline glycoside where the drug is contained in the internal aqueous phase or in the internal surface of the liposomes.

Surprisingly, liposome-complexed anthracycline glycosides prepared in-accordance with the present invention present the same advantage as liposome-encapsulated doxorubicin from U.S. Patent 4,419,348 in which the drug is first complexed and then encapsulated, regarding reduced cardiotoxicity. Thus, in accordance with the present invention, the procedure of preparing the liposomal-encapsulated doxorubicin as well as other anthracycline glycosides is greatly simplified, presenting advantages both in terms of scale-up and stability.

Having described the present invention, reference will now be made to certain examples which are provided solely for purposes of illustration and which are not intended to be limitative.

EXAMPLE 1: Preparation of a cardiolipin-containing liposome composition of the invention

Small unilamellar vesicles were formed by mixing 19.1 μmol cardiolipin, 96.2 μmol phosphatidyl choline and 64.6 μmol cholesterol. After thorough stirring, the mixture is evaporated to dryness in a 50 ml round-bottom flask using a

rotary evaporator. The subsequent dried lipid film is resuspended in 10 ml sterile non-pyrogenic water. After a 30 min. swelling time, the resulting suspension was sonicated (Heat System, W220F) in a fixed temperature bath at 25°C for 15 min. Liposomal preparation is then submitted to lyophilization with the trehalose, a sugar.

The following lipids were used: Phosphatidyl choline (egg), Cardiolipin (bovine), natural and/or synthetic and cholesterol All of these materials are commercially available.

EXAMPLE 2: Preparation of an Anthracycline Cardiolipin Containing liposome composition with Doxorubicin

Complex formation and integration of Doxorubicin into the lipid bilayer membrane of the cardiolipin-containing liposome was achieved prior to the clinical administration by simple vortex mixing of a vial containing 40 mg cardiolipin-liposome lyophilizate and 2.5 ml of a Doxorubicin solution previously prepared in 0.85% NaCl at 2 mg/ml. Vortex mixing is completed for 1 minute and mixture is kept at 37°C for a 15 min. period incubation. Doxorubicin HCl was obtained from Adria Laboratories.

EXAMPLE 3: Determination of the Preferred Conditions of Incubation for Complexing Cardiolipin-containing Liposomes to Doxorubicin

Formation of the complex liposome-doxorubicin was evaluated relative to the time of the incubation at 25°C following the mixing of both components (Figure 1). Complexing index (expressed as percent of amount of doxorubicin complexed to liposomes from initial input dose) was determined following incubation after centrifugation of the mixture at 45,000 rpm for 30 min. at 4°C and measurement of doxorubicin content in subsequent supernatant by spectrophotometry.

The results obtained show that a 15 min. incubation period was enough to obtain a satisfactory complex formation index of 77%. Longer exposure increase slightly the formation of the complex until 92% of the drug complexed after a 24 hr. incubation.

Association capacity of doxorubicin to cardiolipin-liposomes was evaluated for different concentrations of mixture of both components mixed in the same volume (5ml) after an incubation of 15 min. at 37°C at a fixed liposome/doxorubicin weight ratio of 11 (Table 1). The result obtained show that nearly all the drug present in the incubation mixture was complexed to the cardiolipin-containing liposomes revealing an increase of complex formation when the incubation is carried out at 37°C compared to an incubation temperature of 25°C. In addition, results demonstrate that even at a high dilution of both components (20 μg/ml of Dox and 0.22 mg/ml of liposomes) complex-formation is very effective and nearly complete.

EXAMPLE 4: Study of the *in vitro* Drug Delivery Efficiency by Cardiolipin-Liposomes-Complexed Doxorubicin. Effect on Multidrug Resistance.

Resistance to major classes of cytotoxic drugs may emerge in tumor cells from patients treated by chemotherapy. Therefore, multidrug resistance may be one therapeutic obstacle in cancer treatment. It has been shown that liposome-encapsulated doxorubicin may modulate multidrug resistance in cancer cells. (A.R. Thierry, T.J. Jorgensen, D. Forst, I.A. Belli, A. Dritschilo, A. Rahman. Modulation of Multidrug Resistance in Chinese Hamster Cells by Liposome-encapsulated Doxorubigin. Cancer comm. vol. 1 pp. 311-316. (1989)). The capability to increase Doxorubicin activity in multidrug resistant cells was due to the use of a liposonal carrier. This capability was studied when cardiolipin-liposome-complexed doxorubicin. Thus, multidrug resistance

reversal ability bore witness to the integrity or stability of the cardiolipin-liposome-doxorubicin complex.

Clonogenic assay was performed to evaluate modulation of multidrug resistance of free doxorubicin, cardiolipin-liposome-complexed doxorubicin and liposome-encapsulated doxorubicin in MCF-7/ADR and LZ cells which are resistant to doxorubicin. MCF-7/ADR and LZ cells are multidrug resistant cell lines originating from human breast cancer and Chinese hamster fibroblast, respectively.

An accurately known number of cells plated in a $10cm^2$ culture cell were treated for 4 hrs. at different drug concentrations. Following exposure, cells were washed twice with PBS and fresh culture medium was added. The cells were left for a 15 day incubation period to develop colonies. The resulting colonies were then stained with methylene blue and counted. Percent survival of treated cells was determined relative to untreated control. Cytotoxic activity was expressed as IC_{50} which was defined as the concentration of drug resulting in 50% survival of the colonies compared to control.

In Figure 2, cytotoxicity of free doxorubicin, liposome-encapsulated doxorubicin, and the cardiolipin-liposome-complexed doxorubicin were compared using LZ cells. In Dox+Lip I treatment, cells were exposed to cardiolipin-liposome-complexed doxorubicin which was prepared from mixing drug to a concentrate of previously formed cardiolipin-liposomes. Dox+Lip II correspond to cardiolipin-liposome-complexed doxorubicin which was prepared from mixing drug to a cardiolipin-liposome lyophilizate. The three liposomal doxorubicin preparations used in this experiment exhibited comparable cytotoxicity against LZ cells and thus a comparable drug resistance reversal capacity (approximately 9-fold compared to free drug in terms of IC_{50}).

Survival curves of MCF-7/ADR cells treated with free Doxorubicin, empty liposomes, cardiolipin-liposomes and doxorubicin in simultaneous combinations with empty cardiolipin-liposomes are shown in Figure 3. In this experiment cardiolipin-liposomes were complexed with free doxorubicin directly in the culture medium (volume 5 ml) before cell treatment and cardiolipin-liposomes concentration added to the drug was equal to that present at equivalent doxorubicin concentration in liposome-encapsulated doxorubicin. Both liposomal doxorubicin preparations present a comparable cytotoxic effect and thus a comparable drug resistance reversal effect. It was established in the human breast carcinoma resistant MCF-7/ADR cell line that both liposomal doxorubicin preparations have the same cytotoxic activity revealing that the drug in the cardiolipin-liposome-complex is as well integrated as in the liposome-encapsulated drug.

Figure 4 shows the reversal capacity of various treatments in the LZ cells. Generally, reversal capacity to multidrug resistance refers to the level at which drug resistance is overcome and is measured by the ratio of these amounts for free drug/liposome-encapsulated drug. For example, if 5 mg of free drug or 1 mg of liposome-encapsulated drug are required to overcome resistance to a drug, a reversal capacity of 5 is indicated. This would generally indicate that the liposome-encapsulated drug is five times as effective as the free drug in a drug-resistant host. Results demonstrate that increased concentrations of liposome in combination with Doxorubicin, for example substantially enhance the cytotoxic effect of the drug. As shown previously, liposome encapsulated doxorubicin seems to be approximately as cytotoxic as cardiolipin-liposome-complexed doxorubicin when liposome concentration (0.2 mg/ml) added to drug was equal to that present at equivalent drug concentration. For example, when 0.6 mg/ml and 1.0 mg/ml of liposomes (concentration corresponding to 90% and 50% survival for liposome cytotoxicity alone in LZ cells) are added to

doxorubicin the reversal capacity of these treatments are 22 and 28 fold, respectively, showing a higher reversal capacity compared to liposome encapsulated doxorubicin (9-fold).

Evidence of the role of the complexed cardiolipin-liposome-doxorubicin on multidrug resistance is demonstrated in Figure 2. LZ cells were exposed to different concentrations of cardiolipin-liposome-complexed doxorubicin corresponding to a liposome/doxorubicin weight ratio of ii. As previously observed, drug is nearly all complexed to the liposome when incubated at 37°C and the liposomal doxorubicin preparation exerts a cytotoxic effect higher than that of free drug. When vincristine (an alkaloid anticancer agent) is mixed in the same conditions as Doxorubicin to cardiolipin-containing liposomes, no association between drug and liposome was formed and no increase in drug cytotoxicity was observed. The results demonstrate that cardiolipin-liposome complexed specifically doxorubicin and that this association is responsible for the increase of cytotoxicity against multidrug resistant cells.

In addition to the surprising advantages described above, the present invention also affords all of the advantages previously obtainable with liposomal anthracycline glycoside compositions. Notably, reference may be made to U.S. 4,419,348, which is incorporated herein in the entirety. Further, the following experiments illustrate the adaptability and versatility of the present method of making blank cardiolipin containing liposomes and their effective and efficient internalization of the drug inside the liposomes because of the strong affinity of the specific lipid to doxorubicin. The present method of preparing liposomes not only greatly simplifies the industrial manufacturing but actual patient treatment because of the usefulness of the method near the bed side of a patient as has been demonstrated by the spontaneous internalization of doxorubicin to the lyophilized and reconstituted liposomes.

TABLE 1: Combined treatment of Lip with Dox or VCR Cytotoxic Effect in LZ Cells and Binding Capacity of Lip or Dox or VCR

DRUG CONCENTRATION µg/ml	LIP CONCENTRATION in drug cytotoxicity mg/ml	DOX + LIP		VCR + LIP	
		% free drug in the medium after incubation	fold increase in drug cytotoxicity	% free drug in the medium	fold increase in drug cytotoxicity
20	0.22	1.5	1.6	99.3	1.1
50	0.55	4.4	5.9	98.5	1.0
100	1.10	3.5	8.4	98.1	1.1
150	1.65	2.9	34.4	97.9	1.7

The fold increase noted in Table 1 was determined as the ratio of IC_{50} of the drug to that of the combined treatment of the drug and Lip % free drug in the medium after incubation was determined as described above.

Additionally, a most important aspect of the present invention resides in the discovery that very high yields of anthracycline glycoside complexation may be obtained. Generally, complexation (encapsulation) yields of at least 75% anthracycline glycoside may be obtained. However, preferably a yield of at least 95% is obtained.

Generally, the yield of complexation is increased by prolonging the time with which the various constituent ingredients are mixed in the complexation mixture.

Furthermore, although the preferred ratio of cardiolipin used to anthracycline glycoside will vary from glycoside to another, in general a molar ratio of glycoside to cardiolipin of about 3:1 to 1.5:1 is used, preferably, however, about 2:1.

Finally, in accordance with the present invention, it is noted that high yields of encapsulation may be obtained even if very dilute solutions of liposome and anthracycline glycoside or analog thereof are used. For example, solutions of liposome--which are as dilute as 0.5 mg/ml, preferably at least 0.10 mg/ml may be used.

With respect to the solution of anthracycline glycoside or analog thereof, solutions as dilute as 5 mg/ml, preferably 10 mg/ml, may be used.

More preferably, however, a solution concentration of liposomes of at least 0.20 mg/ml is used, and a solution concentration of anthracycline glycoside or analog thereof of at least 15 μg/ml is used.

Of course, higher solution concentrations of each may be used as desired.

Additionally, it is specifically contemplated in the present invention that the same may be practiced with a single anthracycline glycoside or analog thereof or a mixture of two or more anthracycline glycosides or analogs thereof.

Further, in accordance with the present invention, pharmaceutical compositions are provided which contain, at least, one or more liposomal anthracycline glycoside compositions and a pharmaceutically acceptable carrier. It is, further, explicitly contemplated that these pharmaceutical compositions may contain a mixture of different liposomal anthracycline glycosides.

Finally, as indicated above, in accordance with the present invention, it is now possible to obtain an effective treatment against different types of cancer cells by

modulating the ratio of anthracycline glycoside/liposome as a function of the type of cancer cells to be treated.

In general, for any particular type of cancer, malignant cells thereof may be cultured *in vitro* and subjected to various ratios of anthracycline glycoside/liposome in order to determine the most effective ratio. This ratio affording the best results may then be used with the *in vivo* method of treatment for the patient. In using this selection method, conventional culturing techniques may be used for cultivating the malignant cells *in vitro*. Further, this method may be applied to virtually any form of cancer. The many forms of cancer are well known to those skilled in the art.

For example, the present invention may be used to prepare liposomal anthracycline glycoside compositions which may be used in the treatment of carcinomas, sarcomas or leukemia and lymphoma. Generally, carcinoma refers to any cancer of epithelial tissues including body surfaces, digestive tracts and organs originating therefrom. Sarcoma refers generally to any cancer of non-epithelial supportive tissues. Leukemia and lymphoma are non-epithelial malignant tumors which can be included within the group of sarcomas in a broad sense. However, they are cancers of wandering cells appearing in the blood and are considered apart from fixed sarcomas.

Further, as examples of solid tumors, i.e. carcinomas or sarcomas, may be mentioned tumors of the gullet, oral cavity,

stomach, duodenum, small intestine, large intestine, anus, gall bladder, labium, nasopharynx, liver, skin, pancreas, bronchus, lung, mammary gland, uterus, ovary, prostate gland, male reproductive organ and urinary organs, such as the bladder and kidney.

Furthermore, the liposomal anthracycline glycosides of the present invention may include within pharmaceutical compositions which include other ingredients, such as known stabilizers, buffers, surfactants, excipients as well as other active ingredients which are used in the treatment of cancer. Such additional ingredients are well known to those skilled in the art.

The compositions of the present invention may be administered in any manner, such as, for example, intravenously, a intraperitoneally or orally, if desired.

Having described the present invention, it will be apparent that many changes and modifications may be made to the above-described embodiments without departing from the spirit and the scope of the present invention.

Claims

1. A method of preparing a liposomal anthracycline glycoside composition, which comprises:

a) forming cardiolipin-containing liposomes by drying a lipid mixture containing cardiolipin, and then introducing an aqueous solution, and b) mixing the cardiolipin-containing liposomes with a solution of anthracycline glycoside or an analog thereof for a time sufficient to encapsulate said anthracycline glycoside or analog thereof.

2. The method of Claim 1, wherein said anthracycline glycoside is doxorubicin, daunorubicin, or epirubicin.

3. The method of Claim 1, wherein said analog is a deoxy analog of doxorubicin.

4. The method of Claim 1, wherein said lipid mixture comprises at least one neutrally or negatively charged lipid.

5. The method of Claim 3, wherein said at least one lipid comprises phosphatidyl choline, cholesterol, phosphatidyl serine or phosphatidyl glycerol.

6. The method of Claim 1, wherein said cardiolipin-containing liposomes are lyophilized and rehydrated for use.

7. The method of claim 1, wherein said mixing of step b) comprises homogenizing the mixture using a vortex, magnetic stirrer or sonication or combination thereof.

8. The method of Claim 1, wherein at least about 75% of the anthracycline glycoside complexes with the cardiolipin-containing liposomes.

9. The method of Claim 8, wherein at least 95% of the anthracycline glycoside complexes with the cardiolipin-containing liposomes.

10. The method of Claim 1, wherein said mixing is effected for at least about 15 minutes.

11. The method of Claim 10, wherein said mixing is effected for about 24 hours.

12. The method of Claim 1, wherein said solution containing liposomes, and solution of anthracycline glycoside or analog thereof are each dilute solutions.

13. A method of preparing a liposomal anthracycline glycoside composition, which comprises either:

a) rehydrating lyophilized cardiolipin containing liposomes with a solution of anthracycline glycoside, or b) rehydrating the mixture of lyophilized liposomes and

crystalline doxorubicin.

14. A method of selecting a ratio of anthracycline glycoside or analog thereof/liposome for effecting treatment of cancer in a mammal, which comprises:

a) determining a preferred ratio of anthracycline glycoside or analog thereof/liposome for treating cancer cells *in vitro*, and

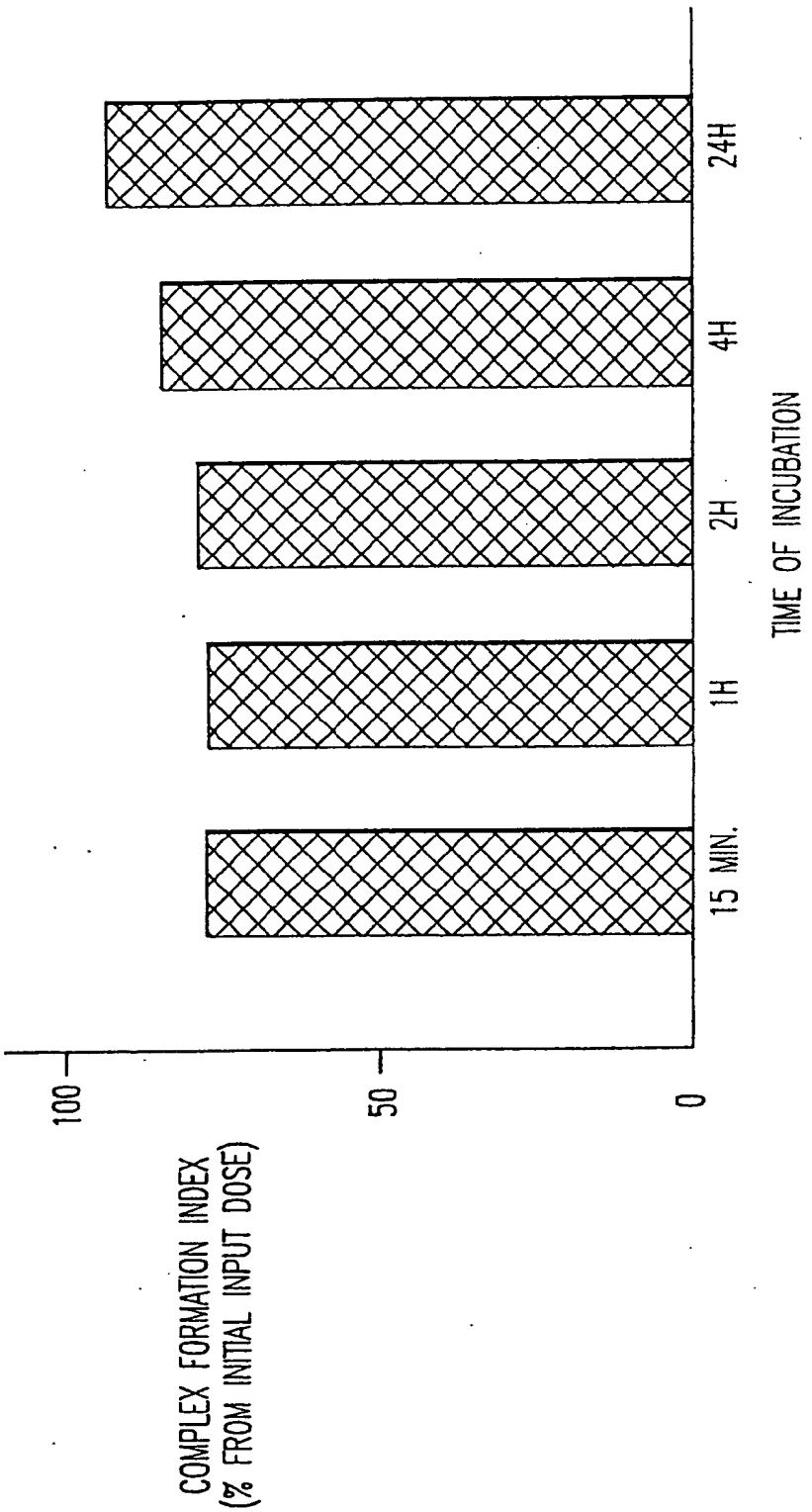
b) applying said ratio to the treatment of cancer cells *in vivo*.

15. The method of Claim 13, wherein said mammal is human.

16. A method of preparing a liposomal anthracycline glycoside composition, which comprises rehydrating lyophilized cardiolipin-containing liposomes with a solution of anthracycline glycoside.

17. A method of preparing a liposomal anthracycline glycoside composition, which comprises rehydrating a mixture of lyophilized liposomes and crystalline doxorubicin.

FIG. 1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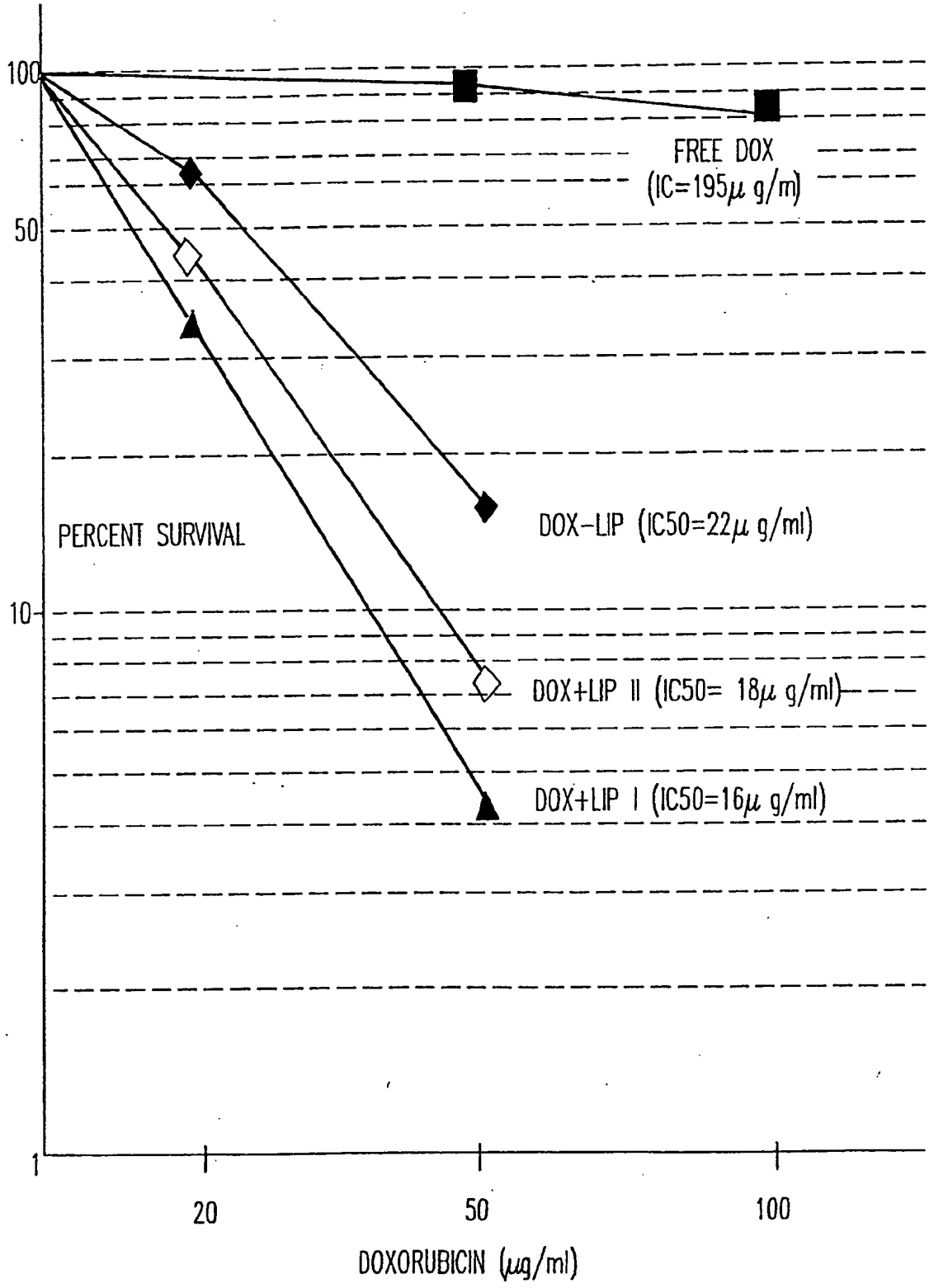
1H
2H
4H
24H
TIME OF INCUBATION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(IC=195μ g/m)
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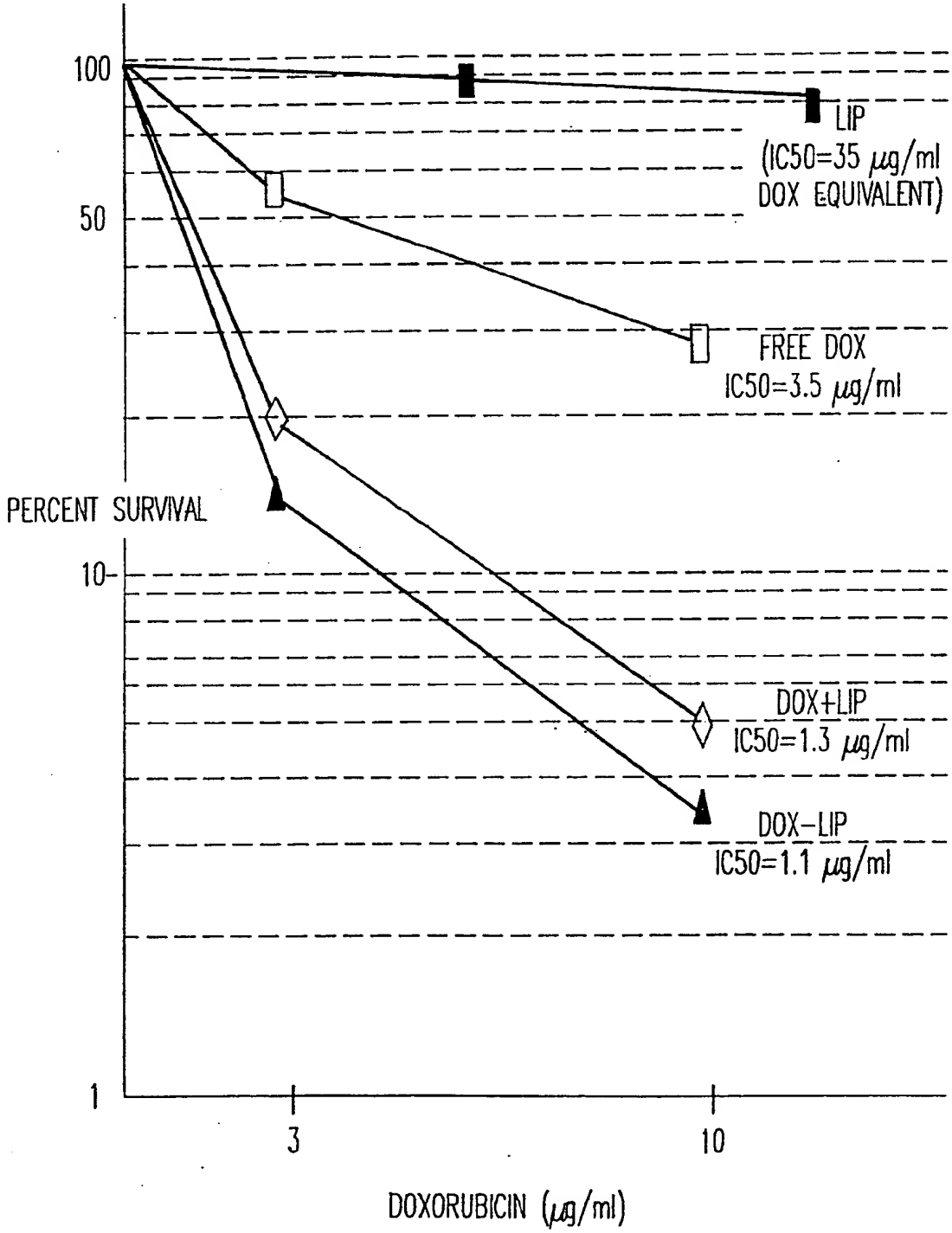
DOXORUBICIN (μg/ml)

FIG. 2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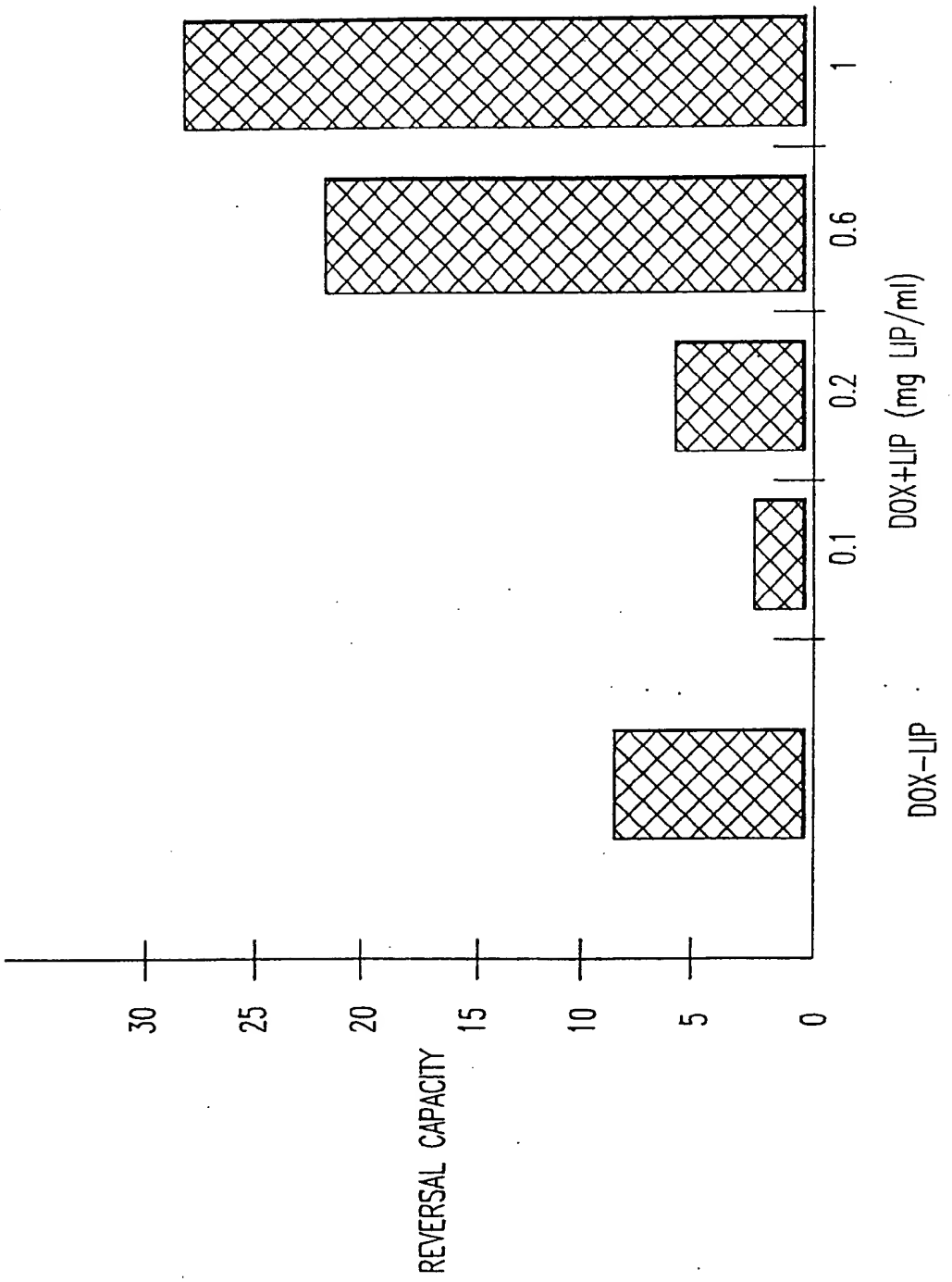
LIP
(IC50=35 μg/ml
DOX EQUIVALENT)
FREE DOX
IC50=3.5 μg/ml
DOX+LIP
IC50=1.3 μg/ml
DOX-LIP
IC50=1.1 μg/ml
3
10
DOXORUBICIN (μg/ml)

FIG. 3

FIG. 4


REVERSAL CAPACITY
0
5
10
15
20
25
30
DOX-LIP
0.1
0.2
0.6
1
DOX+LIP (mg LIP/ml)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08032

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 9/127
US CL :424/450

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/450

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,419,348 (RAHMAN ET AL) 06 DECEMBER 1983; SEE ENTIRE DOCUMENT.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 17 NOVEMBER 1993	Date of mailing of the international search report 17 DEC 1993
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE	Authorized officer JYOTHSNA VENKAT Telephone No. (703) 308-2351

Form PCT/ISA/210 (second sheet)(July 1992)*

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1318188 - THOMAS,SCOTT

Number of Requests in Group:	4	
Approximate Number of Lines:	275	
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WEST'S CALIFORNIA LOCAL COURT RULES PAMPHLETS AND WEST'S CALIFORNIA RULES OF COURT

LOCAL RULES FOR THE UNITED STATES DISTRICT COURT FOR THE NORTHERN DISTRICT OF CALIFORNIA

CIVIL LOCAL RULES

RULE 16. CASE MANAGEMENT AND PRETRIAL CONFERENCES

Current with amendments received through 3-1-1999.

RULE 16-6. DISCLOSURES AND PRETRIAL PROCEEDINGS IN PATENT CASES

(a) Scope. In addition to complying with Civil L.R. 16-5 and FRCiv P 26, in all civil actions filed in this court which include a claim of patent infringement, or which seek a declaratory judgment that a patent is not infringed or is invalid, the parties shall also comply with the disclosure requirements and procedures set forth in Civil L.R. 16-6 through 16-11.

(b) Confidentiality. If any document or information produced under Civil L.R. 16-6--16-11, is deemed confidential by the producing party and if the court has not entered a protective order, until a protective order is issued by the court, the document shall be marked "confidential" by the disclosing party and disclosure of the confidential document or information shall be limited to each party's outside attorney of record. If a party is not represented by outside attorney, disclosure of the confidential document or information shall be limited to a designated "in-house" attorney. The attorney to whom disclosure of a confidential document or information is made under this local rule shall keep it confidential and use it only for purposes of litigating the case.

(c) Certification and Admissibility of Initial Disclosures. All statements, disclosures or charts filed or served in accordance with these local rules must be dated and signed by counsel of record. Counsel's signature shall constitute a certification that to the best of his or her knowledge, information and belief, formed after an inquiry that is reasonable under the circumstances, the information contained in the statement, disclosure or chart is complete and correct at the time it is made.

(d) Duty to Supplement Disclosures. Unless otherwise ordered by the court, disclosures or charts governed by these local rules are subject to the duty of supplementation of FRCivP 26(e).

(e) Admissibility of Disclosures. Unless otherwise ordered by the court, statements, disclosures or charts governed by this local rule shall be admissible as evidence during the course of the action.

Adopted, eff. July 1, 1997.

U. S. Dist. Ct. Rules N.D.Cal., Civil L.R. 16-6

CA R USDCTND Civil L.R. 16-6

END OF DOCUMENT

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RULE 16. CASE MANAGEMENT AND PRETRIAL CONFERENCES

Current with amendments received through 3-1-1999.

RULE 16-7. INITIAL DISCLOSURE OF ASSERTED CLAIMS AND PRIOR ART IN PATENT CASES

(a) Initial Disclosure of Asserted Claims. No later than 45 days after filing a pleading that includes a claim for patent infringement, the party claiming patent infringement, except in those cases described in Civil L.R. 16- 8, must serve on all parties an "Initial Disclosure of Asserted Claims" in conformity with Civil L.R. 16-7(b) and must produce or make available for inspection and copying the documents described in Civil L.R. 16-7(c).

(b) Content of Initial Disclosure of Asserted Claims. Separately for each opposing party, the "Initial Disclosure of Asserted Claims," shall contain the following information:

(1) Each claim of each patent in suit that is allegedly infringed by each opposing party;

(2) Separately for each allegedly infringed claim, each accused apparatus, product, device, process, method, act or other instrumentality ("accused instrumentality") of each opposing party of which the party is aware. This identification shall be as specific as possible. Each product, device and apparatus must be identified by name or model number, if known. Each method or process must be identified by name, if known, or by any product, device or apparatus which, when used, results in the practice of the claimed method or process;

(3) The date of conception and the date of reduction to practice of each asserted claim.

(c) Document Production Accompanying Initial Disclosure of Asserted Claims. At the time of filing the "Initial Disclosure of Asserted Claims," the party claiming patent infringement must produce to each opposing party or make available for inspection and copying all documents relating to:

(1) Any offers to sell each claimed invention prior to the date of application for the patent; and

(2) Research, design, and development of each claimed invention.

(d) Initial Disclosure of Prior Art. No later than 55 days after service upon it of an "Initial Disclosure Of Asserted Claims," each opposing party shall serve on all parties an "Initial Disclosure Of Prior Art" which conforms to Civil L.R. 16-7(e) and must produce or make available for inspection and copying the documents described Civil L.R. 16-7(f).

(e) Content of Initial Disclosure of Prior Art. The Initial Disclosure of Prior Art shall contain the following information:

(1) Each item of prior art that the party contends anticipates the claim or renders it obvious;

(2) For each item of prior art, whether it anticipates the claim or renders it obvious, if a combination of prior art references renders a claim obvious, that combination must be identified.

(3) The identification of prior art must be as specific as possible. Each prior art patent shall be identified by its number, country of origin, and date of issue. Each prior art publication, shall be identified by its title, date of publication and, where feasible, its author and publisher. Prior art under 35 U.S.C. § 102(b) shall be identified by specifying the item offered for sale or publicly used, the date the offer or use took place, and the identity of the person or entity which made the use or which made and received the offer.

(f) Document Production Accompanying Initial Disclosure of Prior Art. At the time of filing the "Disclosure of Prior Art," each opposing party must produce or make available for inspection and copying any source code, specifications, schematics, flow charts, artwork, formulas or other documentation on any accused instrumentality.

Adopted, eff. July 1, 1997.

U. S. Dist. Ct. Rules N.D.Cal., Civil L.R. 16-7

CA R USDCTND Civil L.R. 16-7

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CIVIL LOCAL RULES

RULE 16. CASE MANAGEMENT AND PRETRIAL CONFERENCES

Current with amendments received through 3-1-1999.

RULE 16-9. CLAIM CHART IN PATENT CASE

(a) Service and Content of Claim Chart. No later than 70 days after service upon it of an "Initial Disclosure Of Prior Art," under Civil L.R. 16-7(d) or 16-8, any party claiming patent infringement shall serve upon all parties a "Claim Chart." Separately, with respect to each opposing party against whom a claim of patent infringement is made, the Claim Chart must contain the following information:

(1) Each claim of any patent in suit which the party alleges was infringed;

(2) The identity of each apparatus, product, device, process, method, act or other instrumentality of each opposing party which allegedly infringes each claim;

(3) Whether such infringement is claimed to be literal or under the doctrine of equivalents;

(4) Where each element of each infringed claim is found within each apparatus, product, device, process, method, act or other instrumentality; and

(5) If a party claiming patent infringement wishes to preserve the right to rely on its own apparatus, product, device, process, method, act or other instrumentality as evidence of commercial success, the party must identify, separately for each claim, each such apparatus, product, device, process, method, act or other instrumentality that incorporates or reflects that particular claim.

(b) Response Chart. No later than 60 days after service of upon it of a "Claim Chart," each party opposing a claim of patent infringement, shall serve on all parties a "Response Chart" which must contain the following information:

(1) The identity of each item of prior art that anticipates the claim or renders it obvious. Each prior art patent shall be identified by its number, country of origin, and date of issue. Each prior art publication must be identified by its title, date of publication, and where feasible, author and publisher. Prior art under 35 U.S.C. § 102(b), shall be identified by the item offered for sale or publicly used, the date the offer or use took place, the identity of the person or entity which made the use or which made and received the offer; and

(2) Whether it anticipates the claim or renders it obvious. If a combination of prior art references makes a claim obvious, that combination must be identified;

(3) Where, specifically, within each item of prior art each element of the claim is found;

(4) All grounds of invalidity other than anticipation or obviousness of any of the claims listed in Claimant's Claim Chart. This identification must be as specific as possible. For example, if a best mode defense is raised, the adverse party must set forth with particularity what constitutes the inventor's best mode, specifically citing

information or materials obtained in discovery to the extent feasible. If an enablement defense is raised, the adverse party must set forth with particularity what is lacking in the specification to enable one skilled in the art to make or use the invention; and

(5) If the claimant has alleged willful infringement, the date and a document reference number of each opinion of counsel upon which the party relies to support a defense to the willfulness allegation, including but not limited to, issues of validity, and infringement of any patent in suit.

(c) Amendment to Claim Chart. Amendment of a Claims Chart or a Response Chart may be made only on stipulation of all parties or by order of the court, which shall be entered only upon a showing of excusable subsequent discovery of new information or clearly excusable neglect.

Adopted, eff. July 1, 1997.

U. S. Dist. Ct. Rules N.D.Cal., Civil L.R. 16-9

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RULE 16. CASE MANAGEMENT AND PRETRIAL CONFERENCES

Current with amendments received through 3-1-1999.

RULE 16-10. CLAIM CONSTRUCTION PROCEEDINGS IN PATENT CASES

(a) Proposed Claim Construction Statement. No later than 70 days after service of the "Initial Disclosure of Prior Art," pursuant to Civil L.R. 16- 7(d), each party claiming patent infringement must serve on all parties a "Proposed Claim Construction Statement," which shall contain the following information for each claim in issue:

(1) Identification of any special or uncommon meanings of words or phrases in the claim;

(2) All references from the specification that support, describe, or explain each element of the claim;

(3) All material in the prosecution history that describes or explains each element of the claim; and

(4) Any extrinsic evidence that supports the proposed construction of the claim, including, but not limited to, expert testimony, inventor testimony, dictionary definitions and citations to learned treatises, as permitted by law.

(b) Response to Proposed Claim Construction Statement. No later than 60 days after service upon it of a Proposed Claim Construction Statement, each opposing party must serve on each party a "Response to Proposed Claim Construction Statement." The response shall contain the following information:

(1) Identification of any special or uncommon meanings of words or phrases in the claim in addition to or contrary to those disclosed pursuant to Civil L.R. 16-10(a)(1);

(2) All references from the specification that support, describe, or explain each element of the claim in addition to or contrary to those disclosed pursuant to Civil L.R. 16-10(a)(2);

(3) All material in the prosecution history that describes or explains each element of the claim in addition to or contrary to those disclosed pursuant to Civil L.R. 16-10(a)(3); and

(4) Any extrinsic evidence that supports the proposed construction of the claim, including, but not limited to, expert testimony, inventor testimony, dictionary definitions and citations to learned treatises, as permitted by law.

Adopted, eff. July 1, 1997.

U. S. Dist. Ct. Rules N.D.Cal., Civil L.R. 16-10

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1318188 - THOMAS,SCOTT

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